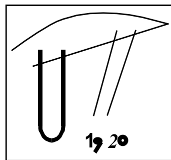

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SOL-GEL IMMOBILIZED ENZYMES ACTIVITY IN ESTERIFICATION AND TRANSESTERIFICATION REACTIONS

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ABSTRACT

Sol-gel immobilization of enzymes, which can improve considerably their properties, is now one of the most used techniques. This involves the entrapment of enzymes within a polymer matrix, in the presence of catalysts and additives. Sol-gel lipase immobilizates are excellent catalysts in esterification and transesterification reactions.

This work presents the results of esterification of lauric acid with 1-octanol, and transesterification of vinyl acetate with 2-pentanol, as model reactions for testing sol-gel immobilized lipases. Silica entrapped lipase-catalyzed transesterifications, which involve chiral substrates, represent an excellent method to produce chiral compounds. Microbial lipases from Candida cylindracea (Sigma) and from Pseudomonas fluorescens (AK Amano) have been used.

Good recovery yields (about 68%) were achieved in the esterification reactions, validating the possibilities of the sol-gel method.

Gas-chromatographic analyses of reaction mixtures from transesterification reactions have been performed using a chiral capillary column. Enantiomeric excess values were in the range of 0.24 - 0.78, and the enantioselectivity of the enzyme were higher following the immobilization.

KEYWORDS:

sol-gel immobilization, microbial lipases, esterification, enantioselective transesterification

1. INTRODUCTION

Industrial bioconversion processes often require high-density, heterogeneous biocatalysts that can be produced in specific forms and should display high activities and long-term stabilities under a range of operating conditions. Biotransformations can be used especially as versatile tools in chiral technologies, alongside conventional methods as asymmetric synthesis or traditional resolution techniques [1].

Lipases are enzymes able to catalyse a variety of reactions, such as esterifications, transesterifications and hydrolysis [2]. Because of their selectivity, lipases are important biocatalysts for asymmetric synthesis based on chiral or racemic substrates and therefore to synthesize chiral compounds [3].

The need for recycling of enzymes and improving their properties led to immobilization techniques. Sol-gel immobilization of enzymes is now one of the most used techniques. The sol-gel process has been known from more than a century as a route to metal-oxide, silica and organosiloxane matrixes of defined porosity, but its importance in protein entrapment was recognized only in the last decade [4,5].

The formation of a sol-gel biocomposite begins with the partial or complete hydrolysis of a suitable precursor to form an aqueous sol. The precursors could be tetraalcoxysilanes, mono-, di-, or tri-alkyl alcoxysilanes, or may contain other functional groups. Then, the hydrolyzed sol precursor is mixed with a buffered aqueous solution containing the biomolecule along with any additive required to adjust the properties of the final product. The polymerization reaction is usually promoted by a change in pH or by the presence of a catalyst. The resulting gelation of the sol precursor is accompanied by the entrapment of the biomolecule. The initial gels are soft, having high water content. Aging the wet silica network promotes further condensation along with elimination of alcohol and water. Finally, drying of the aged material result in further cross-liking reactions, loss of most of the interstitial water and shrinkage of the pores. The dried gel is called xerogel [6].

Encapsulation in organically modified silicates, prepared by hydrolysis of alkyl-substituted silane compounds in the presence of the enzyme was demonstrated to be a very efficient method to obtain immobilized lipases with high activity. This technique has been accomplished by using hydrophobic silica matrices prepared from alkyl-substituted organic silane precursors, typically alkyltrimethoxysilanes $RSi(OCH_3)_3$. It is plausible that a displacement of a lid covering the active centre of the lipase takes place and, consequently, an activation of the enzyme occurs. Further, the encapsulation of the activated lipase is accompanied by the interaction of the lipophylic domain of the biocatalyst with the alkyl groups of the silica matrix [7,8]. This technique seems to be generic for lipases, but it was demonstrated that very small change in the experimental conditions (temperature, solvent, concentrations, nature and concentration of the catalyst, presence of additives or templating compounds, as well as external factors) can drastically modify the properties of the obtained solid preparate [9-11]. Therefore, the experimental protocol must be optimized for every application.

The sol-gel technique was used in this work to generate hydrophobic silica matrices by hydrolysis of silane compounds as tetraethoxysilane (TEOS), methyltrietoxysilane (MTEOS), ethyltrietoxysilane (EtTEOS) and phenyltrietoxysilane (PhTEOS) at different molar ratios, in the presence of additives such as polyethylene glycols (PEG). The polycondensation reaction was promoted by using NaF catalyst or by switch of the pH to basic range with ammonium solution. The immobilised derivatives have been characterized with respect to their catalytic activities in esterification and transesterification reactions.

2. MATERIALS AND METHODS

Commercial *Candida cylindracea* lipase was purchased from Sigma Chemical Co.(USA), and *Pseudomonas fluorescens* lipase AK was from Amano Enzyme Inc. (Japan).

The silane precursors methyltrietoxysilane (MTEOS), ethyltrietoxysilane (EtTEOS) and phenyltrietoxysilane (PhTEOS) were purchased from Aldrich, and tetraethoxysilane (TEOS) was from Fluka. Polyethylene glycol (PEG, M=20,000 from Fluka) was used as stabilising agent. Other materials used: ethanol (99.2%, Chimopar), isopropyl alcohol (99.7%, Chimopar), sodium fluoride (Fluka), 1-octanol (95%, Fluka), 2-pentanol (Fluka), lauric acid (Fluka), vinyl acetate (Fluka), n-hexane (98%, Merck), n-hexadecane (99%, Merck), isooctane (99.5%, Serva), n-decane (95%, Riedel de Haën), were of analytical grade and have been used without further purification.

3. IMMOBILIZATION OF CANDIDA CYLINDRACEA LIPASE

The methodology published by Reetz *et al*. [8] has been adapted and modified in some cases, according to our previously established protocol for proteolytic enzymes [12]. *Candida cylindracea* lipase was suspended (100mg/mL) in 0.1 M TRIS/HCl (pH=8.0) buffer solution, shaked at room temperature for 30min, filtered, and the filtrate was used for immobilization. In a 4mL glass vial, 780µL *Candida cylindracea* filtrate, 200 µL PEG(MW 20000, 4%w/v), 100 µL NaF 1M, and 200 µL isopropyl alcohol were mixed, and the amounts of silane precursors given in the Table 1 were added. In further experiments, a sol precursor (from the mentioned silanes and hydrochloric acid in ethanol) was obtained 24 hours before immobilization and mixed with the enzyme, additive, and ammonium solutions.

The two-phase mixture was thoroughly mixed on a magnetic stirrer for 30min, and the closed vial was allowed to stand at room temperature for 24h. The bulk gel was washed with distilled water or pH 8 buffer solutions, isopropyl alcohol and hexane and dried at room temperature. Finally, it was crushed to a fine powder and kept in a refrigerator.

Immobilization of Pseudomonas fluorescens AK lipase

In a 4mL glass vial, 50mg AK Amano lipase was suspended in 390µL TRIS/HCl buffer solution (0.1 m, pH=8.0) vigorously stirred for 5min. Then, 200 µL PEG (MW 20,000, 4%w/v), 100 µL NaF 1M, 200 µL isopropyl alcohol, and the silane precursors (amounts given in Table 2) were added. Further, the same protocol as described for the Candida cylindracea lipase was used.

General procedure for determination of the lipase activity in esterification reactions

The free or sol-gel immobilized Candida cylindracea lipase (40mg) was placed in a small magnetically stirred glass reactor with lauric acid (0.2 mmol), 1-octanol (0.4 mmol), n-hexadecane (30µL, internal standard for chromatographic analysis), isooctane (2 mL, solvent), at 40ºC. At

defined intervals, usually after 24 h, samples have been taken and the yield of formed product was determined by gas chromatography using a BPX 5 capillary column (SGE Australia Pty. Ltd., 15 m x 0,32 mm). A Dani 86.10 gas chromatograph equipped with flame ionization detector was used. Esterification activity at 24h was calculated based on ester yields and it was expressed as micromoles of ester formed per hour per mg of immobilized enzyme.

$$\text{Activity} = \frac{\mu\text{moles}_{\text{ester}}}{24 \cdot \text{m}} \qquad \text{U/mg}$$

m = amount of lipase used in the reaction (usually 40mg)

The recovery yield (in %) of the enzymatic activity following immobilization was determined as the ratio of total activity of the immobilized enzyme and total activity of the free enzyme.

$$\text{Recovery yield} = \frac{\text{Total activity immobilized enzyme}}{\text{Total activity free enzyme}} \cdot 100 \qquad (\%)$$

General procedure for evaluating immobilized lipases as catalysts in the kinetic resolution of a chiral alcohol

A small magnetically stirred glass reactor was charged with a mixture of 2-pentanol (1mmol), vinyl acetate (3 mmol), n-decane (30 µL, internal standard for chromatographic analysis), hexane (2 mL, solvent) and free (5 mg) or sol-gel immobilized (50 mg) AK Amano lipase. The mixture was incubated at 40ºC, with continuous agitation. Samples taken at different intervals were analysed by gas chromatography using a BPX 5 capillary column (SGE Australia Pty. Ltd., 15 m x 0,32 mm) to determine the alcohol conversion, and using a CYDEX-B chiral capillary column (SGE Australia Pty. Ltd., 30 m x 0,32 mm), to determine the enantiomeric excess. The recovery yields of the enzyme activity have been also calculated, as previously mentioned. Transesterification activities were usually calculated using the values of alcohol conversions after 24 h.

Based on Kaslauskas rule [3] it was assumed that Amano AK lipase is (R)-specific in the acylation reaction of 2-pentanol, therefore the main formed ester is the (R)-ester. Enantiomeric excess based on the resulted product has been expressed as:

$$\text{e.e.}_{\text{ester}} = \frac{\%\,\text{ester(R)} - \%\,\text{ester(S)}}{\%\,\text{ester(R)} + \%\,\text{ester(S)}}$$

4. RESULTS AND DISCUSSION

Esterification studies with sol-gel entrapped Candida cylindracea lipase

Esterification activities for immobilized lipases are often more important than hydrolytic activities, as the main objective is to utilise the

enzyme as biocatalyst in synthetic applications. Ester yield vs. time dependences of the esterification reactions between lauric acid and n-octanol are shown in Figure 1. We have also calculated the esterification activities and the activity recovery yields (Table 1).

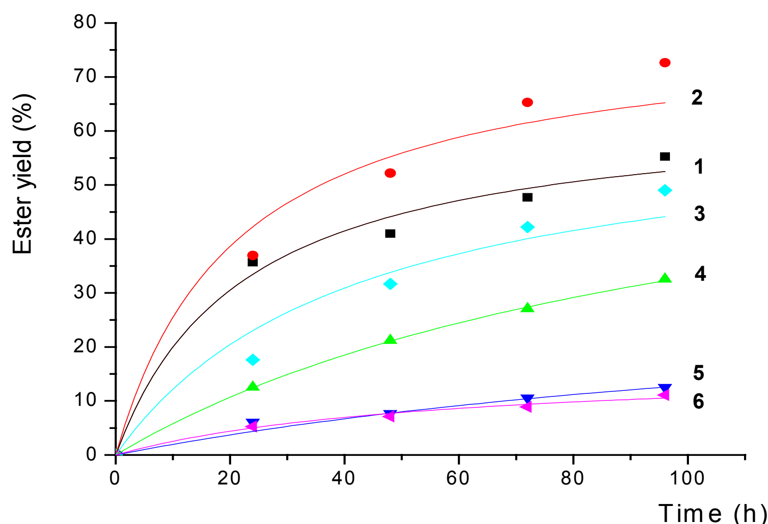

Figure 1. Synthesis of octyl laurate using free and sol-gel entrapped *Candida cylindracea* lipase. The significance of the synthesis number is given in Table 1.

Table 1. Esterification activities and recovery yields at different silane precursor ratios

Synthesis No.	Silane precursors (molar ratio)	Esterification activity (μmoli/h g_{enz})	Recovery yield (%)
-	No (free enzyme)	297,5	100,00
1	PhTEOS /TEOS (3:1) (1.086 mL PhTEOS + 0.335 mL TEOS)	36,6	68,26
2	MTEOS /TEOS (5:1) (0.996 mL MTEOS + 0.223 mL TEOS)	11,0	30,10
3	EtTEOS /TEOS (3:1) (0.967 mL EtTEOS + 0.335 mL TEOS)	-	< 1
4	EtTEOS /TEOS (1:1) 2 mL Sol (4.07 mL EtTEOS, 4.23 mL TEOS, 8 mL EtOH, 3.25 mL H_2O, 0.1 mL HCl)	26,1	17,14
5	MTEOS /TEOS (3:1) (0.896 mL MTEOS + 0.335 mL TEOS)	12,9	37,56
6	MTEOS/TEOS (1:1) (0.596 mL MTEOS + 0.670 mL TEOS)	56,1	52,10

In the classical sol-gel process, hydrolysis and condensation reactions lead to nano-sized gel particles, which then cross-link to form amorphous SiO_2 gel. For lipase encapsulation, this process is usually performed with tetramethoxysilane and alkyltrimethoxysilanes, using NaF catalyst, as it was described by Reetz et al. [7,8]. The main problem arising from this methodology is the uncontrollable heat dissipation during the process and the formation of methanol, a possible inhibitor of some lipases, as by-product of the condensation. To overcome the above-mentioned drawbacks, we used ethoxysilane derivatives as starting materials. The gelation time was considerably higher than reported for methoxysilane derivatives, resulting in better heat dissipation, but in some experiments gelation was difficult to be accomplished. However, the lipase entrapped in gels formed from TEOS showed low activity, probably

determined by the gel shrinkage during the xerogel formation. To obtain sol-gels with less shrinkage and higher activity, silanes containing alkyl- and aryl-groups have been used as precursors and polyethylene glycols as additives. In these conditions we got good recovery yields of the enzyme activity after immobilization, up to 68% related to the free enzyme.

The nature of the silane precursor has considerably influenced on the lipase activity. Unexpectedly, the highest recovery yield was obtained with PhTEOS as precursor, but using the same precursor at different molar ratios failed in attending appropriate gelation. The network forming ability of this precursor is lower than for alkyl-containing silanes. The highest activity of the immobilized lipase was observed when MTEOS and TEOS in equimolar amounts have been used. The molar ratio of TMOS related to TEOS also influenced the activity, and decreased with an increasing TMOS/TEOS molar ratio. Even if hydrophobic alkyl-modified silanes are known as better environment for lipase entrapment, it seems that more than 50% molar percentage of the alkyl-containing precursor is not recommended.

Enantioselective transesterifications with sol-gel encapsulated lipase

Enantioselectivity of sol-gel immobilized enzymes can be influenced by the immobilization method. It is important to obtain an enhancement of the optical selectivity following immobilization, as enantioselectivity is one of the main advantages of enzymatic biotransformations. Acylation of 2-pentanol was achieved with high enantioselectivity using sol-gel encapsulated Amano AK lipase (Figure 2 and Table 2). The analysis was performed on a CYDEX-B chiral capillary column, as described in Materials and methods.

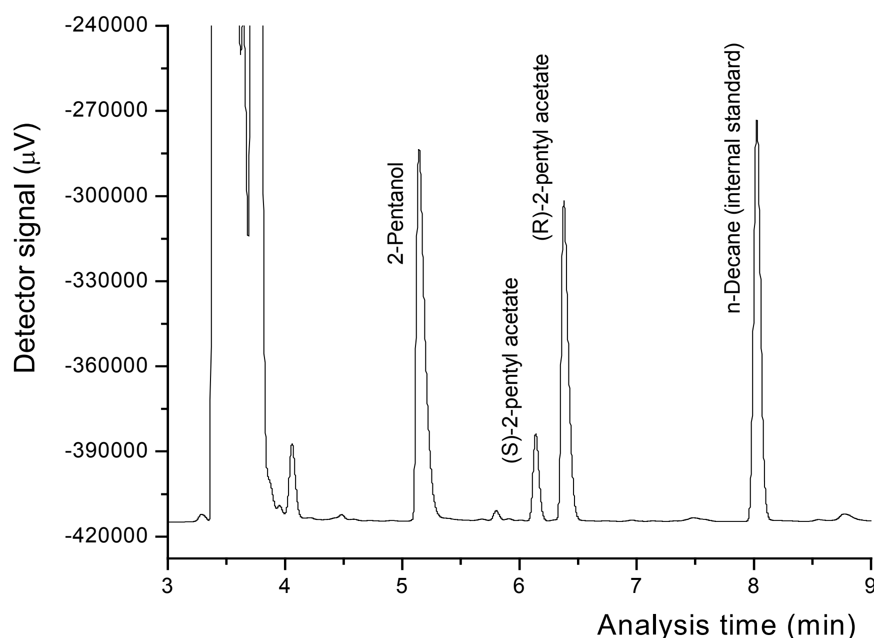

Figure 2. Chromatogram of the transesterification reaction product of vinyl acetate with 2-pentanol (synthesis No. 7), after 24 h reaction time.

Table 2. Transesterification of vinyl acetate with 2-pentanol, catalyzed by free and sol-gel entrapped lipase from Pseudomonas flourescens (Amano AK)

Synthesis No.	Silane precursors (molar ratio)	Transesterification activity µmoli/h mg	Recovery yield %	Conversion* %	e.e.*
-	Free enzyme	4,939	-	64,6	49
7	MTEOS/TEOS (5:1) (0.498 mL MTEOS + 0.111 mL TEOS)	0,462	23,0	42,2	81
7[a]	MTEOS/TEOS (5:1) (0.498 mL MTEOS + 0.111 mL TEOS)	0,220	-	28,7	85
8	MTEOS/TEOS (1:1) (0.298 mL MTEOS + 0.335 mL TEOS)	0,178	15,1	23,3	79

*calculated after 24 h reaction time
[a]reused enzyme

These experiments were carried out with MTEOS and TEOS as silane precursors. In this case, is recommended to utilise a higher molar percent of alkyl-containing precursor. The best results were obtained using 5:1 molar ratio of MTEOS/TEOS, NaF as catalyst and polyethylene glycol as additive. In industrial applications, the quality of an expected kinetic resolution depends not only upon the achieved enantioselectivity, but also on the activity and possibility of reusing the enzyme. We therefore studied the possibility of recycling the most active encapsulated enzyme (synthesis 7a), even if it was not possible to achieve an optimization from a few number of experiments. It can be pointed out than in acylation reaction involving a chiral substrate, 2-pentanol, enhanced enantioselectivity based on kinetic resolution has been achieved following encapsulation of the lipase. In the recycling experiment, the enantioselectivity of the reaction remained the same, but the activity of the enzyme was reduced by 50% (Figure 3). The recovery yields of the enzymatic activity were between 23-42%. The entrapment of lipase in the sol-gel matrix lead to an increased enantioselectivity in acylation reactions with vinyl acetate, with a consistent maintenance of the enzyme activity.

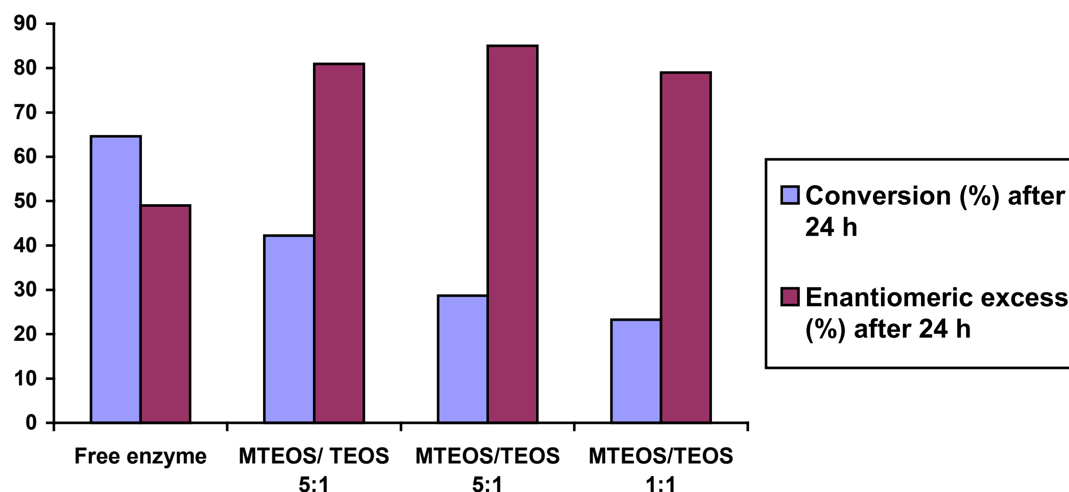

Figure 3. Conversion and enantiomeric excess in the acylation reaction of 2-pentanol, catalyzed by *Pseudomonas fluorescens* (Amano AK) lipase, as a function of silica precursor molar ratio.

5. CONCLUSIONS

The immobilised derivatives of *Candida cylindracea* lipase by the sol-gel technique are excellent catalysts both in esterification and transesterification reactions. It is possible to achieve high lipase loading as well as consistent protein immobilization efficiency and improved enantioselectivity. The highest activity of the immobilized enzyme was strongly dependent on the nature and molar ratio of the silane precursors. The sol-gel technique offers remarkable opportunities to obtain bioactive nanocomposite materials with enhanced operational properties as a new generation of biocatalysts.

REFERENCES

1. K. Drauz, H. Waldmann (1995) *Enzyme Catalysis in Organic Synthesis*, VCH, Weinheim.
2. F. Peter, G. Preda, A. Chiriac (1998) *Chemistry Monographs Series of the West University of Timisoara*, *No. 19*.
3. U.T. Bornscheuer, R.J. Kazlauskas (1999) *Hydrolases in Organic Synthesis*, Wiley-VCH, Weinheim.
4. I. Gill, A. Ballesteros A. (2000) *Trends Biotech.* 18, 282-296.
5. I. Gill, E. Pastor, A. Ballesteros (1999) *J. Am. Chem. Soc*. 121, 9487-9496.
6. W. Jin, J.D. Brennan (2002) *Anal. Chim. Acta* 461, 1-36.
7. M.T. Reetz, A. Zonta, J. Simpelkamp (1995) *Angew. Chem. Int. Ed. Engl.* 34, 301-303.
8. M.T. Reetz, P. Tielmann, W. Wisenhöfer, W. Könen, A. Zonta (2003) *Adv. Synth. Catal.* 345, 717-728.
9. S. Furukawa, T. Ono, H. Ijima, K. Kawakami (2001) *J. Mol. Catal. B: Enzym.* 15, 65-70.
10. J.-P. Chen, W.-S. Lin (2003), *Enzym. Microb. Technol*. 32, 801-811.
11. C.M.F. Soares, O.A. dos Santos, J.E. Olivo, H.F. de Castro, F.F. de Moraes, G.M. Zanin (2004) *J. Mol. Catal. B: Enzym.* 29, 69-79.
12. G. Preda, C. Savii, F. Peter, A. Chiriac, M. Dragomirescu, C. Ionescu, I. Stan, M. Popovici, P. Prisecaru, S. Bacardzieva, S. Subrt (2002) *International Congress on Biocatalysis*, Hamburg.